REMARKS

Status of Claims

This paper cancels claim 10, amends claim 8-9 and 18-20, and adds new claims 23-38. Applicants reserve the right to prosecute canceled subject matter in related applications. After the amendments set forth above are entered, claims 2-9, 11, and 14-38 are pending and under examination.

Support for all claim amendments and new claims is found generally throughout the Specification, the pending and cancelled claims, and the claims as filed. Specifically, support for the amendment to claim 20, specifying that the method monitors an amplification reaction in real-time is found at page 1, lines 17-23, Example 3 (pages 9-10), and Figure 1. Applicant submits that the term "real-time" is both exemplified in the Specification and well-known in the art as a method for detecting a target nucleic acid by "the continuous collection of fluorescent signal from [a] polymerase chain reaction throughout [the] cycles." See Exhibit A: Dorak, Glossary of Real-time PCR Terms, http://dorakmt.tripod.com/genetics/glosrt.html; Exhibit B: PCRLinks.com, http://www.pcrlinks.com/glossary/R.htm, citing Higuchi et al., 1993; and Exhibit C: Hunt, Real Time PCR, Microbiology and Immunology On-line, University of South Carolina, School of Medicine, hyyp://pathmicro.med.sc.edu/pcr/realtime-home.htm. The most salient feature of real-time PCR is the simultaneous quantification and amplification of the target nucleic acid. University of Ottawa, Glossary, http://www.rfcom.ca/glossary/index.shtml. See Exhibit D.

Support for new independent claim 23 is found, for example, in Example 3 (pages 9-10) and Figure 1, which demonstrate a method of monitoring the accumulation of target nucleic acid during an amplification reaction by measuring the fluorescent signal associated with the accumulation of the amplified target nucleic acid during multiple PCR cycles. No new matter is introduced by these new claims and amendments.

Interview Summary

Applicants wish to thank Examiners Tung and Stzelecka for the courtesies extended to Applicant's representatives during the interview of September 16, 2008. The substance of the interview is reflected in the following summary and the claim amendments and remarks contained herein.

As an initial matter, Applicant notes that substantially the same claims as currently under examination were previously rejected over Heller and that rejection was withdrawn. See, Office Action mailed May 22, 2006 and Applicant's Reply of July 17, 2006. Based on the discussion between Examiners Tung and Stzelecka and Applicant's representatives during the interview, it appears that the Heller rejection was reasserted on the belief that the examined claims broadly encompass a method for detecting a target nucleic acid, including in a static, non-cycling environment.

Applicant strongly disagrees with the Examiner's interpretation of the examined claims. During the interview, Applicant's representatives argued that the examined claims are limited to real-time monitoring of an amplification reaction because claim 20 requires "monitoring said target nucleic acid during said amplification;" a feature not disclosed in Heller. Examiners Tung and Stzelecka asserted their interpretation of independent claim 20, alleging that the claims are not so limited. No agreement was reached on an acceptable claim amendment that would immediately bring the claims into condition for allowance, however, the Examiners agreed that, in principle, Heller may be overcome by limiting the claimed method to real-time detection.

Applicant continues to traverse the Examiner's interpretation of claim 20, as examined. However, in order to expedite prosecution, the claims are amended herein to be unambiguously limited to detection methods involving simultaneous nucleic acid amplification and detection. In view of the long pendency of this case (filed in 1998), and the reassertion of a previously traversed rejection, Applicant sincerely requests an early indication that the amended claims are in condition for allowance.

Rejections under 35 USC § 102

Claims 2-7 and 19-22 stand rejected as anticipated by Heller (U.S. Patent 5,565,322) as evidenced by Mullis et al. (U.S. Patent 4,965,188). The Examiner alleges that Heller discloses a method for monitoring nucleic acid amplification comprising amplifying a target nucleic acid and monitoring said target nucleic acid during said amplification using two oligonucleotide probes having the same properties specified in independent claim 20. Office Action mailed July 16, 2008 at p. 2, ¶ 4. Applicant respectfully traverses this rejection.

As discussed during the interview of September 16, 2008, the Examiner alleges that the phrase "monitoring said target nucleic acid during said amplification" does not limit the claims to real-time monitoring. Applicant strenuously disagrees with the Examiner's interpretation of this phrase. However, in order to expedite prosecution, claim 20 has been amended to specifically require that that the monitoring of the amplification reaction is performed in real-time. Additionally, new independent claim 23 requires that the fluorescence of the first probe fluorophore is detected during a plurality of cycles. Thus, like the amendment to claim 20, claim 23 also requires that fluorescence detection occur contemporaneously with the amplification reaction. Applicant respectfully submits that these amendments fully address the rejection over Heller predicated on the Examiner's belief that the examined claims encompassed detection under both cycling (i.e., amplification) and non-cycling (static) conditions.

Heller does not anticipate the instant claims because Heller does not reasonably describe or enable a method for monitoring nucleic acid amplification during amplification. The Examiner specifically identifies several passages in Heller to support this rejection. However, each of these teachings, at most, describes a static (i.e., non-cycling) detection assay and contains no mention of the elements required for an amplification reaction including a polymerase, deoxynucleotide triphosphates, the use a cycling mechanism characteristic of PCR, or a real-time acquisition of signal during multiple amplification steps.

At column 19, lines 20-56 (relied upon by the Examiner), Heller states:

> Thus a diagnostic method for detecting the presence of a preselected nucleic acid sequence in a nucleic acid-containing sample is contemplated comprising the steps of:
>
> a) admixing:
>
> (i) a polynucleotide having (1) at least two donor chromophores operatively linked to a polynucleotide by linker arms, such that the chromophores are positioned by linkage along the length of the polynucleotide at a donor--donor transfer distance, and (2) at least one fluorescing acceptor chromophore operatively linked to the polynucleotide by a linker arm, such that the fluorescing acceptor chromophore is positioned by linkage at a donor-acceptor transfer distance from at least one of the donor chromophores, wherein the polynucleotide has a nucleotide sequence that is preselected as to be complementary to the preselected "target" nucleic acid sequence; with
>
> (ii) a nucleic acid-containing sample containing the preselected nucleic acid base ("target") sequence to form a hybridization reaction admixture;
>
> (b) subjecting the hybridization reaction admixture to hybridization conditions for a time period sufficient for the polynucleotide to hybridize to the target sequence and form a donor chromophore containing- and acceptor chromophore containing-hybridized nucleic acid duplex;
>
> (c) exciting the donor chromophore in the nucleic acid duplex formed in step (b) by exposing the donor chromophore to sufficient photonic energy to induce emission of photonic energy from the acceptor chromophore; and
>
> (d) detecting the presence of photonic energy emitted from the excited acceptor chromophore, thereby detecting the presence of the preselected nucleic acid sequence in the sample.
>
> Heller at col. 19, ll. 8-40.

In the above detailed methodology, Heller describes no polymerase, no mention of deoxynucleotide triphosphates, no mention to use a cycling mechanism characteristic of PCR, and no mention real-time acquisition of signal during multiple amplification steps. Based on the total absence of the elements required for real-time nucleic acid detection, this passage can only

refer to a *post facto* detection methodology in which the full complement of target nucleic acid is present at the outset of detection.

The Examiner also relies on the Heller at col. 21, ll. 28-35 in support of this anticipation reaction. However, this passage also fails to suggest real-time monitoring of an amplification reaction. Applicant respectfully directs the Examiner to the full context of the cited passage at col. 20, line 57 through col. 21, line 46. Here, Heller describes the various formats in which the disclosed detection methodology may be applicable, including heterogeneous and homogenous formats. Heller at col. 20, ll. 57-67. Heterogeneous formats include an insoluble matrix (e.g., solid support) to which the target nucleic acids are bound; whereas, homogenous formats contain soluble target nucleic acids. Although Heller mentions PCR (a homogeneous format) as a possible source of target nucleic acid, Heller makes clear that the target nucleic acid detection is performed under static, non-cycling conditions. Heller states:

> Where the nucleic acid containing a target sequence is in a double-stranded (ds) form, it is preferred to first denature the dsDNA, as by heating or alkali treatment, prior to conducting the hybridization reaction. The denaturation of the dsDNA can be carried out prior to admixture with a polynucleotide to be hybridized, or can be carried out after the admixture of the dsDNA with the polynucleotide. Where the polynucleotide itself is provided as a double-stranded molecule, it too can be denatured prior to admixture in a hybridization reaction mixture, or can be denatured concurrently therewith the target-containing dsDNA.
>
> Heller at col. 21, ll. 36-46.

It is clear from the context of this passage that, at most, Heller describes only preferred hybridization conditions (i.e., buffer, temperature, etc.) for the above static assay method. There is no indication that target nucleic acid detection occurs in real-time during an amplification reaction. In fact, several of the proposed methodologies are inconsistent with real-time detection. For example, in the above passage, Heller suggests the denaturation of dsDNA be performed by alkali treatment. This denaturation method is incompatible with a real-time PCR detection methodology which necessarily uses heat denaturation. Furthermore, Heller suggests that the

dsDNA may be denatured prior to admixture with the probe polynucleotide, or that the probe polynucleotide, if double-stranded, may be denatured prior to admixture with the hybridization reaction. These methods are also incompatible with a real-time monitoring system in which all components must be simultaneously present during denaturation and reannealing. This passage is typical of the entire Heller patent in being silent to real-time amplification and the specifics of a real-time assay including failing to mention a polymerase, deoxynucleotide triphosphates, a cycling mechanism characteristic of PCR, or use of real-time acquisition of signal during multiple amplification steps. Nothing in this passage demononstrates any conception of a real-time detection system, as currently claimed.

Finally, the Examiner cites to Heller's example illustrated in Figure 4 and the accompanying text at col. 6, ll. 38-52 and col. 28, ll. 14-39. This example, like the rest of the Heller specification, fails to disclose any real-time amplification monitoring methods. In fact, this example specifically describes a target nucleic acid detection under non-cycling conditions.

In describing this assay system, Heller states:

> FIG 4 shows the homogeneous assay procedure. This procedure can be carried [out] using aqueous buffers common to the art of hybridization... The target DNA is either already present or now added to the system.
>
> Heller at col. 28, ll. 14-22 (emphasis added).

Heller's instruction that "[t]he target DNA is either already present or now added to the system" mandates detection under non-cycling conditions. This instruction is incompatible with real-time amplification monitoring in which the target DNA, if present, is progressively generated and accumulates over time.

In summary, Heller does not describe, enable, or contemplate a method for monitoring of target nucleic acid during amplification. Throughout the specification, Heller describes detection methodologies only in the context of a static, non-cycling system. There is no disclosure of the

elements necessary to perform real-time monitoring. Thus, Heller does not anticipate the instant claims.

The Examiner relies on Mullis et al. as evidence that PCR amplification uses a thermostable polymerase and a pair of primers. Office Action at page 4, ¶ 1. Applicant does not dispute the Examiner's characterization of Mullis et al., but note that this reference does not address the deficiencies in Heller as an anticipatory reference. Specifically, nothing in Mullis et al. evidences that Heller performed real-time amplification monitoring. Accordingly, this rejection is traversed and should be withdrawn.

Rejections under 35 USC § 103

Rejection of Claims 8-10

Claims 8-10 stand rejected as obvious over Heller as evidenced by Mullis et al. The Examiner applies Heller as above, noting that Heller's probes have a different lengths, but acknowledges that Heller does not disclose that the second probe is at least three nucleotides shorter than the first probe. The Examiner alleges that it is routine for the artisan to optimize the probe lengths, rendering claims 8-10 obvious. Applicant respectfully traverses this rejection.

Applicant submits that it is irrelevant whether or not it is routine to optimize the probe lengths in the manner alleged by the Examiner. For the reasons discussed above, Heller fails to provide a method for real-time monitoring of target nucleic acids during an amplification reaction. Thus, this rejection is traversed and should be withdrawn.

Rejection of Claim 11

Claim 11 stands rejected as obvious over Heller, as evidenced by Mullis et al., in view of Di Cesare (U.S. Patent 5,716,784). The Examiner applies Heller as above but acknowledges that Heller fails to disclose a complementary probe pair having the specified difference in melting temperature. The Examiner alleges that Di Cesare provides complementary analytical and

detection probes having melting temperatures that differ by more than two degrees and that it is obvious to use probes having these properties in the method of Heller. Applicant respectfully traverses this rejection.

Di Cesare does not remedy the deficiencies of Heller. As discussed above, Heller fails to provide a method for real-time monitoring of target nucleic acids during an amplifieation reaction. Di Cesare, like Heller, also fails to provide such a method. Di Cesare instructs the artisan to perform an amplification reaction in its entirety and then assess the amplification product for the target sequence of interest. Di Cesare at col. 6, ll. 28-51. Thus, regardless of the physieal properties of the Di Cesare probes, this combination of references fails to teach Applicant's claimed real-time monitoring method. Accordingly, this rejection is traversed and should be withdrawn.

Rejection of Claims 14-18

Claims 14-18 stands rejeeted as obvious over Heller, as evidenced by Mullis et al., in view of Hiroaki et al. (EP 0461863). The Examiner applies Heller as above and acknowledges that Heller does not disclose that the target polynucleotide comprises hepatitis C virus genome, the probe having the sequence of SEQ ID NO: 3 and 4 and the primer having the sequence of SEQ ID NO: 1 and 2. The Examiner alleges that Hiroaki discloses a nucleotide which comprises SEQ ID NO:1 and 3 and the complementary sequence of SEQ ID NO: 2 and base pair 1-17 of SEQ ID NO: 4, and that it would have been obvious to use these probes and primers in the method of Heller. Applicant respectfully traverses this rejection.

Hiroaki et al. do not remedy the deficiencies of Heller. Specifically, Hiroaki makes no reference whatsoever to monitoring of amplified nucleic acid during amplification. Thus, for this reason alone, the alleged combination of Heller and Hiroaki et al. fails to establish a *prima facie* case of obviousness because the combination fails to teach or suggest all of the claim limitations. Accordingly, this rejection is traversed and should be withdrawn.

CONCLUSION

Applicant respectfully submits that the pending claims are in condition for allowance. An early notice to that effect is earnestly solicited. Should any matters remain outstanding, the Examiner is encouraged to contact the undersigned at the telephone number listed below so that they may be resolved without the need for a written action.

The Commissioner is hereby authorized to charge any fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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GLOSSARY OF REAL-TIME PCR TERMS

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Address for bookmark: http://www.dorak.info/genetics/glosrt.html

Absolute quantification: The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a **standard curve** (as in determination of viral copy number). (Absolute Quantification Page by Pfaffl).

Allelic discrimination assay: Assays designed to type for gene variants. Either differentially labeled (TaqMan®) probes (one for each variant) or a single probe and melting curve analysis can be used for this purpose. Alternative methods include dsDNA-binding dyes (in combination with melting curve analysis). TaqMan®-based allelic discrimination assays are analyzed by differences in threshold cycles or by endpoint fluorescence value for each allele. The results are plotted by fluorescence intensity or by C_t values for each allele at X and Y axes (see Osgood-McWeeney, 2000 and Figures 3-5 in Hu & Chen for examples). See ABI Allelic Discrimination with TaqMan® Probes and Getting Started Guides for ABI 7000 & 7900HT, LightScanner® and Amplifluor® SNPs Genotyping System.

Amplicon: The amplified sequence of DNA in the PCR process.

Amplification plot: The plot of cycle number versus fluorescence signal which correlates with the initial amount of target nucleic acid during the exponential phase of PCR.

Anchor & reporter probes: Two partnering LightCycler (hybridizing) probes that hybridize on the target sequence in close proximity. The anchor probe (donor) emits fluorescence to excite the reporter probe (acceptor) to initiate **FRET**. In allelic discrimination assays, it is important that the reporter probe spans the mutation and has a lower Tm than the anchor probe.

Baseline: The initial cycles of PCR during which there is little change in fluorescence signal (usually cycles 3 to 15).

Baseline value: During PCR, changing reaction conditions and environment can influence fluorescence. In general, the level of fluorescence in any one well corresponds to the amount of target present. Fluorescence levels may fluctuate due to changes in the reaction medium creating a background signal. The background signal is most evident during the initial cycles of PCR prior to significant accumulation of the target amplicon. During these early PCR cycles, the background signal in all wells is used to determine the 'baseline fluorescence' across the entire reaction plate. The goal of data analysis is to determine when target amplification is sufficiently above the background signal, facilitating more accurate measurement of fluorescence.

Calibrator: A single reference sample used as the basis for relative-fold increase in expression studies (assuming constant reaction efficiency). This calibrator should be included in each assay.

Coefficient of variation (CV): Used as a measure of experimental variation. It is important that a linear value (e.g., copy numbers) is used to calculate the CV (but not C_t values which are logarithmic). Intra-assay CV quantifies the amount of error seen within the same assay (in duplicates) and inter-assay CV quantifies the error between separate assays.

C_t (threshold cycle): Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated. Also called crossing point (C_p) in LightCycler terminology.

Derivative curve: This curve is used in Tm analysis. It has the temperature in the x axis and the negative derivative of fluorescence (F) with respect to temperature (T), shown as dF/dT, on the y axis.

The reproducibility of a derivative melting curve is high with a standard deviation of only 0.1 ^{o}C between runs.

dsDNA-binding agent: A molecule that emits fluorescence when bound to dsDNA. The prototype is SYBR® Green I. In real-time PCR, the fluorescence intensity increases proportionally to dsDNA (amplicon) concentration. The problem with DNA-binding agents is that they bind to all dsDNA products: specific amplicon or non-specific products (misprimed targets and primer-dimers included). For this reason, analysis using DNA-binding agents is usually coupled with melting analysis.

Dynamic range: The range of initial template concentrations over which accurate C_t values are obtained. If endogenous control is used for DDC_t quantitation method, dynamic ranges of target and control should be comparable. In absolute quantitation, interpolation within this range is accurate but extrapolation beyond the dynamic range should be avoided. The larger the dynamic range, the greater the ability to detect samples with high and low copy number in the same run.

Efficiency of the reaction: The efficiency of the reaction can be calculated by the following equation: $E = 10^{(-1/slope)} - 1$. The efficiency of the PCR should be 90-100% meaning doubling of the amplicon at each cycle. This corresponds to a slope of –3.1 to –3.6 in the C_t vs log-template amount standard curve. In order to obtain accurate and reproducible results, reactions should have efficiency as close to 100% as possible (e.g., two-fold increase of amplicon at each cycle), and in any case, efficiency should be similar for both target and reference (normalizer, calibrator, endogenous control, internal control). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, presence of inhibitors, secondary structure and primer design. Although valid data can be obtained that fall outside of the efficiency range, if it is < 0.90, the quantitative real-time PCR should be further optimized or alternative amplicons designed (see Efficiency Determination).

End-point analysis: As opposed to quantitative analysis using the data collected during exponential phase of PCR, real-time applications can also be used to collect end-point data for qualitative assays. These are either allelic discrimination assays (genotyping) or absence/presence assays (pathogen detection).

Endogenous control: This is an RNA or DNA that is naturally present in each experimental sample. By using an invariant endogenous control as an active 'reference', quantitation of a messenger RNA (mRNA) target can be normalized for differences in the amount of total RNA added to each reaction and correct for sample-to-sample variations in reverse transcriptase PCR efficiency. See ABI TaqMan Human Endogenous Control Plate; TATAA Biocenter Endogenous Control Gene Panel; geNorm kit; Ambion: 18S RNA as an Internal Control; Ambion: GAPDH, β-actin, cyclophilin, 18S RNA as internal controls; EXPOLDB: The most constantly expressed housekeeping genes; algorithms to select the best endogenous controls: geNORM (Vandesompele, 2002), NormFinder (Andersen, 2004), and qBase*Plus* (Hellemans, 2007).

Exogenous control: This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference dye (usually ROX) in order to normalize for non-PCR-related fluctuations in fluorescence signal.

FAM: 6-carboxy fluorescein. Most commonly used reporter dye at the 5' end of a TaqMan® probe.

Fast PCR: A modified PCR protocol that allows shortening of overall reaction time to less than the typical 90 minutes (usually 40 minutes or less) thanks to recent developments in amplicon design, reagent chemistry, thermocycling conditions as well as the PCR machines with fast ramping rates. See Biocompare Tutorials > Fast PCR (text).

Fluorescence resonance energy transfer (FRET): The interaction between the electronic excited states of two dye molecules. The excitation is transferred from one (the donor) dye molecule to the other (the acceptor) dye molecule. FRET is distance-dependent and occurs when the donor and the acceptor dye are in close proximity.

High resolution melting (HRM) curve analysis: See **Melting curve (dissociation) analysis**.

Housekeeping gene: Genes that are widely expressed in abundance and are usually used as

reference genes for normalization in real-time PCR with the assumption of 'constant expression'. The current trend is first to check which housekeeping genes are suitable for the target cell or tissue and then to use more than one of them in normalization in qPCR assays. See for EXPOLDB: The most constantly expressed housekeeping genes housekeeping genes showing the least inter-individual difference in their expression levels.

Hybridization probe: One of the main fluorescence-monitoring systems for DNA amplification. LightCycler probes are hybridization probes and are not hydrolyzed by Taq Polymerase. For this reason, melting curve analysis is possible with hybridization probes. See Wittwer, 1997 and Hybridization Probe Chemistry for details.

Hydrolysis probe: One of the main fluorescence-monitoring systems for DNA amplification. TaqMan® probes are an example. These kinds of probes are hydrolyzed by the 5' endonuclease activity of Taq Polymerase during PCR. See Wittwer, 1997 for details.

Internal positive control (IPC): An exogenous IPC can be added to a multiplex assay or run on its own to monitor the presence of inhibitors in the template. Most commonly the IPC is added to the PCR master mix to determine whether inhibitory substances are present in the mix. Alternatively, it can be added at the point of specimen collection or prior to nucleic acid extraction to monitor sample stability and extraction efficiency, respectively.

LATE (Linear After The Exponential)-PCR: A new form of asymmetric PCR that uses primer pairs deliberately designed for use at unequal concentrations (Pierce, 2003; Sanchez, 2004). Unlike typical asymmetric PCR, LATE-PCR, amplification is efficient due to improved primer design (Pierce, 2005). LATE-PCR begins with an exponential phase in which amplification efficiency is similar to that of symmetric PCR. Once the limiting primer is depleted, the reaction abruptly switches to linear amplification, and the single-stranded product is made for many additional thermal cycles. LATE-PCR consistently generates strong signals because the absence of product strand reannealing permits unhindered hybridization of the molecular beacon to its target strand and continued accumulation of that strand beyond the cycle at which symmetric reactions typically plateau. By eliminating the exponential phase, LATE-PCR generates less error scatter among replicates. When used in conjunction with molecular beacons, LATE-PCR results in increased signal intensity and reduced sample variation. These features are particularly useful for real-time PCR initiated with single cells. LATE-PCR has been used to directly amplify ssDNA for pyrosequencing (Salk, 2006). See also Bonetta, 2005.

Light-up probe: The light-up probe is a peptide nucleic acid (PNA) oligomer to which an asymmetric cyanine dye thiazole orange (a single reporter dye) is tethered. Upon hybridization the thiazole orange moiety interacts with the nucleic acid bases and the probe becomes brightly (up to 50-fold) fluorescent (Svanvik, 2000a; 2000b & 2001;Isacsson, 2000; Wolffs, 2001). Being based on an uncharged analog (PNA), the light-up probe hybridizes faster and binds target DNA much stronger than oligonucleotide-based probes. See also LightUp Technologies.

Linear View: Amplification plot view displayed using exact ΔRn values on the Y-axis. The alternative is the log-view, which expands the initiation of exponential amplification phase (and also the baseline period activity). Either can be used for threshold setting.

Locked Nucleic Acid (LNA®) Probes: A new generation of sequence-specific probes designed using LNA (a novel nucleic acid analogue), which has enhanced hybridization performance and biological stability (Koch, 2003; Tolstrup, 2003; Johnson, 2004). LNA has also been used in primers to increase sensitivity (Latorra, 2003). See also web brochures by Proligo; Exiqon; IDT; Gene Link; PCR: Replicating Success (Moore, 2005).

Log-dilution: Serial dilutions in powers of 10 (10, 100, 1000 etc).

Log-view: See **Linear View**.

LUX™ (Light Upon eXtension) primers: Created by Invitrogen, LUX™ primer sets include a self-quenched fluorogenic primer and a corresponding unlabeled primer. The labeled primer has a short sequence tail of 4–6 nucleotides on the 5′ end that is complementary to the 3′ end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore. When the primer is incorporated into double-stranded DNA during PCR, the fluorophore is dequenched and the signal increases by up to ten-fold. By eliminating the need for a quencher dye, the LUX™ primers reduce the cost (LUX™ vs TaqMan®).

Melting curve (dissociation) analysis: Every piece of dsDNA has a melting point (Tm) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order, G:C content and Watson-Crick pairing. When DNA-binding dyes are used, as the fragment is heated, a sudden decrease in fluorescence is detected when Tm is reached (due to dissociation of DNA strands and release of the dye). This point is determined from the inflection point of the melting curve or the melting peak of the derivative plot (what is meant by derivative plot is the negative first-derivative of the melting curve). The same analysis can be performed when hybridization probes are used as they are still intact after PCR. As hydrolysis probes (e.g., TaqMan®) are cleaved during the PCR reaction, no melting curve analysis possible if they are used (because of their specificity, there is no need either). Mismatch between a hybridization probe and the target results in a lower Tm. Melting curve analysis can be used in known and unknown (new) mutation analysis as a new mutation will create an additional peak or change the peak area. See Ririe, 1997 for details of melting curve analysis. High-resolution melting curve analysis can be achieved on dedicated instruments like Idaho Technology's LightScanner® or on Corbett's Rotor-Gene 6000.

Minor groove binders (MGBs): These dsDNA-binding agents are attached to the 3' end of TaqMan® probes to increase the Tm value (by stabilization of hybridization) and to design shorter probes. Longer probes reduce design flexibility and are less sensitive to mismatch discrimination. MGBs also reduce background fluorescence and increase dynamic range due to increased efficiency of reporter quenching (these probes use non-fluorescent quenchers at the 3'end). By allowing the use of shorter probes with higher Tm values, MGBs enhances mismatch discrimination in genotyping assays. See ABI Allelic Discrimination with TaqMan® Probes.

Minus reverse transcriptase control (– RTC): A quantitative real-time PCR control sample that contains the starting RNA and all other components for one-step reaction but no reverse transcriptase. Any amplification suggests genomic DNA contamination.

Molecular beacons: These hairpin probes consist of a sequence-specific loop region flanked by two inverted repeats. Reporter and quencher dyes are attached to each end of the molecule and remain in close contact unless sequence-specific binding occurs and reporter emission (FRET) occurs. See How it Works.

Monte Carlo effect: Problems with reproducible quantification of low abundance targets (<1000 copies) by qPCR. It is a limitation of PCR amplification from small amounts of any complex template due to differences in amplification efficiency between individual templates in an amplifying cDNA population. The Monte Carlo effect is dependent upon template concentration; the lower the abundance of any template, the less likely its true abundance will be reflected in the amplified product. Originally described by Karrer, 1995; see Bustin & Nolan, 2004 for details.

Multiplexing: Simultaneous analysis of more than one target. Specific quantification of multiple targets that are amplified within a reaction can be performed using a differentially labeled primer or probes. Amplicon or probe melting curve analysis allows multiplexing in allelic discrimination if a dsDNA-binding dye is used as the detection chemistry.

Normalization: A control gene that is expressed at a constant level is used to normalize the gene expression results for variable template amount or template quality. If RNA quantitation can be done accurately, normalization might be done using total RNA amount used in the reaction. The use of multiple housekeeping genes that are most appropriate for the target cell or tissue is the most optimal means for normalization. This normalization is performed by the experimenter and should not be mixed up with the normalization of fluorescence signal using the passive reference dye (usually ROX) performed by the equipment.

Nucleic acid sequence based amplification (NASBA): NASBA is an isothermal nucleic acid amplification procedure based on target-specific primers and probes, and the coordinated activity of THREE enzymes: AMV reverse transcriptase, RNase H and T7 RNA polymerase. NASBA allows direct detection of viral RNA by nucleic acid amplification. For examples, see Loens, 2003; Guichon, 2004.

No amplification controls (NAC, a minus enzyme control): In mRNA analysis, NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. If cDNA or genomic DNA is used as a template, a reaction mixture lacking Taq polymerase can be included in the assay as NAC. No product should be synthesized in the NTC or NAC. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heating block of the thermal cycler.

No template controls (NTC, a minus sample control): NTC includes all of the RT-PCR reagents except the RNA template. No product should be synthesized in the NTC or NAC; if a product is amplified, this indicates contamination (fluorescent or PCR products) or presence of genomic DNA in the RNA sample. NTC is not equivalent to H_2O controls and H_2O controls are not used in qPCR experiments.

Normalized amount of target: A unitless number that can be used to compare the relative amount of target in different samples.

Nucleic acid target: (also called "target template") - DNA or RNA sequence that is going to be amplified.

Passive reference (reference dye): A fluorescence dye that provides an internal reference to which the reporter dye signal can be normalized during data analysis by the software. This type of normalization is necessary to correct for fluctuations from well to well caused by changes in concentration or volume. **ROX** is the most commonly used passive reference dye.

Peltier element: The element used for heating and cooling in a qPCR machine. Peltier coolers (in ABI machines) use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air flow or mechanical transition between blocks of different temperatures to cycle the samples.

Platform: Refers to hardware that performs real-time PCR. For a current list of available machines, see Michael Pfaffl's page & Biocompare.

PNA (peptide nucleic acid oligomer): See **light-up probe**.

Primer Express® Software: A primer design algorithm by ABI. It designs TaqMan® primer and probe sets to be used at standard conditions of ABI real-time PCR equipment. See Designing TaqMan MGB Probe and Primer Sets for Gene Expression Using Primer Express Software v.2.0 and ABI Taqman Primer/Probe Design using Primer Express.

Quencher: The molecule that absorbs the emission of fluorescent reporter when in close vicinity. Most commonly used quenchers include TAMRA, DABCYL and BHQ. The quenchers are usually at the 3' end of a dual-labeled fluorescent probe. Quencher dye is also called acceptor.

R: In illustrations of real-time PCR principles, 'R' represents fluorescent **R**eporter (fluorochrome).

r coefficient: Correlation coefficient, which is used to analyze a standard curve (ten-fold dilutions plotted against C_t values) obtained by linear regression analysis. It should be ≥ 0.99 for gene quantitation analysis. It takes values between zero and -1 for negative correlation and between zero and +1 for positive correlations.

R^2 coefficient: Usually mixed up with 'r' but this is R-squared (also called coefficient of determination). This coefficient only takes values between zero and +1. R^2 is used to assess the fit of the standard curve to the data points plotted. The closer the value to 1, the better the fit.

Rapid-cycle PCR: A powerful fast PCR technique for nucleic acid amplification and analysis that is completed in less than half an hour. Samples amplified by rapid-cycle PCR are immediately analyzed by melting curve analysis in the same instrument. In the presence of fluorescent hybridization probes, melting curves provide 'dynamic dot blots' for fine sequence analysis, including SNPs. Leading instruments that perform rapid-cycle PCR are RapidCycler2 (Idaho Technology) and LightCycler (Roche).

Real-time PCR: The continuous collection of fluorescent signal from polymerase chain reaction throughout cycles.

Reference: A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification.

Reference dye: Used in all reactions to obtain normalized reporter signal (Rn) adjusted for well-to-well variations by the analysis software. The most common passive reference dye is **ROX** and is usually included in the master mix. Not all instruments require the use of a reference dye (see Table 1 in Real-Time PCR by Qiagen).

Reporter dye (fluorophore): The fluorescent dye used to monitor amplicon accumulation. This can be attached to a specific probe or can be a dsDNA-binding agent (see for example **SYBR® Green I**). For specifications of common reporters, see Table 1 and Figure 1 in Real-Time PCR by Qiagen.

Relative quantitation: A relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as relative increase or decrease -compared to the baseline level- in gene expression in response to a treatment or in time etc). Includes comparative C_t ($\Delta\Delta C_t$) and relative-fold methods. (Relative Quantification Page by Pfaffl).

Ribosomal RNA (rRNA): Commonly used as a normalizer in quantitative real-time RNA. It is not considered ideal due to its expression levels, transcription by a different RNA polymerase and possible imbalances in relative rRNA-to-mRNA content in different cell types.

Rn (normalized reporter signal): The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Rn+ is the Rn value of a reaction containing all components, including the template and Rn– is the Rn value of an unreacted sample. The Rn– value can be obtained from the early cycles of a real-time PCR run (those cycles prior to a significant increase in fluorescence), or a reaction that does not contain any template.

ΔRn (delta Rn, dRn): The magnitude of the fluorescence signal generated during the PCR at each time point. The ΔRn value is determined by the following formula: (Rn+) – (Rn–).

ROX: 6-carboxy-X-rhodamine. Most commonly used passive reference dye for normalization of reporter signal. The emission recorded from ROX during the baseline cycles (usually 3 to 15) is used to normalize the emission recorded from the reporter due to amplification in later cycles. The use of ROX improves the results by compensating for small fluorescent fluctuations such as bubbles and well-to-well variations that may occur in the plate. Not using ROX or not designating it as the passive reference dye in the analysis may cause trailing of the clusters in the allelic discrimination plot.

Scorpion: A fluorescence detection system consists of a detection probe with the upstream primer with a fluorophore at the 5' end, followed by a complementary stem-loop structure also containing the specific probe sequence, quencher dye and a PCR primer on the 3' end. Between the primer and its tail (the probe), a blocking agent (DNA spacer, hexaethylene glycol) is placed. This structure makes the sequence-specific priming and probing a unimolecular event that creates enough specificity for allelic discrimination assays. See How it Works and Scorpion Technology.

Slope: Mathematically calculated slope of standard curve, e.g., the plot of C_t values against logarithm of ten-fold dilutions of target nucleic acid. This slope is used for efficiency calculation. Ideally, the slope should be –3.3 (–3.1 to –3.6), which corresponds to 100% efficiency (precisely 1.0092) or two-fold (precisely, 2.0092) amplification at each cycle. Also called gradient. See Stratagene Slope to Efficiency Calculator.

Standard: A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, a standard curve is created from which the quantity of an unknown sample can be calculated.

Standard curve: Obtained by plotting C_t values against log-transformed concentrations of serial ten-fold dilutions of the target nucleic acid. Standard curve is obtained for quantitative PCR and the range of concentrations included should cover the expected unknown concentrations range. It is used to find out the dynamic range of the target (and/or normalizer), to calculate the slope (therefore, efficiency), r and R^2 coefficients and also to help with quantitation.

Sunrise™ primers: Originally created by Oncor, sunrise™ primers are similar to molecular beacons. They are self-complementary primers that dissociate through the synthesis of the complementary strand and produce fluorescence signals. See also **LUX primers**.

SYBR® Green I: A fluorogenic minor groove binding dye that emits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. It is used as a cheaper alternative in real-time PCR applications. It does not bind to ssDNA but because of the lack of sequence specificity it binds to any dsDNA product. Its use usually requires melting curve analysis to assure specificity of the results (and if multiplexing is attempted). See Morrison, 1998 and How it Works.

TAMRA: 6-carboxy-terta-methyl-rhodamine. Most commonly used quencher at the 3' end of a TaqMan® probe.

TaqMan® probe: A dual-labeled specific hydrolysis probe designed to bind to a target sequence with a fluorescent reporter dye at one end (5') and a quencher at the other (3'). Assays using Taqman probes are also called 5' nuclease assays. See How it Works.

Threshold: Usually 10X the standard deviation of Rn for the early PCR cycles (baseline). The threshold should be set in the region associated with an exponential growth of PCR product (which may be easier is the log-view of the amplification plot is used). It is assigned for each run to calculate the C_t value for each amplification.
Unknown: A sample containing an unknown quantity of template. This is the sample of interest (experimental sample as opposed to positive controls or standards) whose quantity is being determined.

M.Tevfik Dorak, MD PhD

Last updated on 22 August 2007

Genetics Real-Time PCR Homepage

RACE-PCR (rapid amplification of cDNA ends)

- RACE-PCR is an anchor PCR modification of RT-PCR. The rationale is to amplify sequences between a single previously characterized region in the mRNA (cDNA) and an anchor sequence that is coupled to the 5 or the 3 end. A primer is designed from the known internal sequence and the second primer is selected from the relevant anchor sequence
Definition from:
Human Molecular Genetics 2
Tom Strachan & Andrew P. Read, 1999

RAPD (Randomly amplified polymorphic DNA)

- A technique for amplifying anonymous stretches of DNA, using PCR with arbitrary primers.
Definition from:
http://www.igd.cornell.edu/MolecularMarkers/Glossary.pdf

Real Time PCR (Originally described as "**Kinetic PCR**" by Higuchi et al. in 1993)

- Real-time PCR is so named because it detects and measures the amplification of target nucleic acids as they are produced. Real-time PCR requires the use of primers similar to those used in conventional PCR. However, unlike conventional PCR, real-time PCR uses an oligonucleotide probe labeled with fluorescent dyes or an alternative fluorescent detection

chemistry, and a thermocycler equipped with the ability to measure fluorescence. ...
Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples

- Real-Time PCR, also called quantitative (real-time) PCR or Real-Time Quantitative PCR (RTQ-PCR), is a method of simultaneous DNA quantification and amplification. DNA is specifically amplified by polymerase chain reaction. After each round of amplification, the DNA is quantified. Common methods of quantification include the use of fluorescent dyes that intercalate with double-strand DNA and modified DNA oligonucleotides (called probes) that fluoresce when hybridized with a complementary DNA. ...
Definition from:
http://en.wikipedia.org/wiki/Real-Time_PCR

- A technique designed to detect and quantify sequence-specific PCR products as they accumulate in 'real-time' during the PCR amplification process.
Definition from:
http://www.nature.com/nrg/journal/v6/n2/glossary/nrg1525_glossary.html

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Rep-PCR

- Is a type of polymerase chain reaction that targets the repetitive sequences in bacterial genomes using specific primers that are designed complementary to bacterial interspersed repetitive sequences
Definition from:
Lactobacillus Genotyping by Fluorophore - Enhanced Repetitive PCR (FERP) and Capillary Electrophoresis
Ania Szary,2001

Restriction enzyme

- These are enzymes (endonucleases, more specifically) which recognize a specific, short sequence of DNA and cut the DNA at that point. Different restriction enzymes recognize and cut different sequences. There are hundreds of different restriction enzymes available commercially. Many restriction enzymes leave "sticky ends" when they cut, which are available to bind with other "sticky ends" left by the same enzymer. Restriction enzymes are a vital tool in genetics, since they allow cutting (and pasting) of DNA
Definition from:
http://www.acad.carleton.edu/curricular/BIOL/classes/bio125/recdna/glossary.html

- A protein that recognizes specific, short nucleotide sequences and cuts DNA at those sites. Bacteria contain over 400 such enzymes that recognize and cut more than 100 different DNA sequences
Definition from:
Genome Glossary

- An endonuclease that will recognise a specific target sequence and cut the

DNA chain at that point.
Definition from:
http://www.igd.cornell.edu/MolecularMarkers/Glossary.pdf

Restriction site

- The specific nucleotide sequence of DNA at which a particular restriction enzyme cuts the DNA.
Definition from:
http://www.igd.cornell.edu/MolecularMarkers/Glossary.pdf

- A sequence of DNA that is recognized by an endonuclease (a protein that cuts DNA) as a site at which the DNA is to be cut.
Definition from:
Genetests

Reverse transcriptase

-RNA-dependent DNA polymerase - An enzyme that uses an RNA molecule as a template for the synthesis of a complementary DNA (cDNA) strand
Definition from:
http://www.qimr.edu.au/qimr_glossary.html

- A reverse transcriptase, also known as RNA-directed DNA polymerase, is a DNA polymerase enzyme that transcribes single-stranded RNA into double-stranded DNA. Normal transcription involves the synthesis of RNA from DNA, hence reverse transcription is the reverse of this, as it synthesises DNA from RNA
Definition from:
Reverse transcriptase, Wikipedia, The Free Encyclopedia

- An enzyme used by retroviruses to form a complementary DNA sequence (cDNA) from their RNA. The resulting DNA is then inserted into the chromosome of the host cell.
Definition from:
Genome Glossary

Reverse Transcription

- The copying of an RNA molecule back into its DNA complement. The enzymes that perform this function are called reverse transcriptases. Reverse transcription is is used naturally by retroviruses to insert themselves into an organism's genome. Artifically-induced reverse transcription is a useful technique for translating unstable mRNA molecules into stable cDNA.
Definition from:
Glossary of Biotechnology Terms

- The process of copying information found in RNA into DNA.

Definition from:
http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-R/reverse_transcription.html

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RFLP (Restriction fragment length polymorphism)

- Variation in DNA fragment banding patterns of electrophoresed restriction digests of DNA from different individuals of a species. Often due to the presence of a restriction enzyme cleavage site at one place in the genome in one individual and the absence of that specific site in another individual.
Definition from:
http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-R/RFLP.html

- Variation between individuals in DNA fragment sizes cut by specific restriction enzymes; polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. RFLPs usually are caused by mutation at a cutting site.
Definition from:
Genome Glossary

- A genetic polymorphism with respect to the observed length of a restriction fragment. RFLPs can result from single nucleotide polymorphisms as well as from insertions, deletions, or microsatellite expansions
Definition from:
Mouse Genome Informatics

RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

- A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.
Definition from:
http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-R/RT-PCR.html

- A method of amplifying mRNA by first synthesizing cDNA with reverse transcriptase, then amplifying the cDNA using PCR. A positive result is evidence of a particular mRNA, and hence of gene expression, in a sample.
Definition from:
Mouse Genome Informatics

- A two-step process. First, complementary DNA (cDNA) is made from an RNA template, using a reverse transcriptase enzyme, and then some of it is used in a PCR reaction to produce large quantities.
Definition from:
http://www.qimr.edu.au/qimr_glossary.html#R

A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z

REAL TIME PCR
Dr Margaret Hunt

MICROBIOLOGY AND IMMUNOLOGY ON-LINE
University of South Carolina School of Medicine

PowerPoint version of this page

To see larger images, click on the image which will be enlarged in a pop-up window. You must close this window before opening another. This does not yet apply to the first twelve images. These appear on a new page.

FEEDBACK
Please use this address

REAL TIME PCR

Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length, that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (Taq polymerase) that was isolated from *Thermus aquaticus*, a bacterium that grows in hot pools; as a result it is not necessary to add new polymerase in every round of amplification. After several (often about 40) rounds of amplification, the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain. For reasons that will be outlined below, this method of analysis is at best semi-quantitative and, in many cases, the amount of product is not related to the amount of input DNA making this type of PCR a qualitative tool for detecting the presence or absence of a particular DNA. In order to measure messenger RNA (mRNA), the method was extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA) which was then amplified by PCR and, again analyzed by agarose gel electrophoresis. In many cases this method has been used to measure the levels of a particular mRNA under different conditions but the method is actually even less quantitative than PCR of DNA because of the extra reverse transcriptase step. Reverse transcriptase-PCR analysis of mRNA is often referred to as "RT-PCR" which is unfortunate as it can be confused with "real time-PCR".

USEFUL LINKS
Real Time PCR Research
Real time PCR at Wikipedia

Abstract of the original PCR paper

As we shall see below, one reason that makes reverse transcriptase-PCR, as usually practiced, non-quantitative is that ethidium bromide is a rather insensitive stain. Methods such as competitive PCR were developed to make the method more quantitative but they are very cumbersome and time-consuming to perform. Thus, real-time PCR (or reverse transcriptase real-time PCR) was developed.

First, let us review reverse transcriptase PCR in more detail

Polymerase chain reaction (PCR) allows the exponential copying of part of a DNA molecule using a DNA polymerase enzyme that is tolerant to elevated temperatures.

1. mRNA is copied to cDNA by reverse transcriptase using an oligo dT primer (random oligomers may also be used). In real-time PCR, we usually use a reverse transcriptase that has an endo H activity. This removes the mRNA allowing the second strand of DNA to be formed. A PCR mix is then set up which includes a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides and a suitable buffer.

2. cDNA is denatured at more than 90 degrees (~94 degrees) so that the two strands separate. The sample is cooled to 50 to 60 degrees and specific primers are annealed that are complementary to a site on each strand. The primers sites may be up to 600 bases apart but are often about 100 bases apart, especially when real-time PCR is used.

3. The temperature is raised to 72 degrees and the heat-stable Taq DNA polymerase extends the DNA from the primers. Now we have four cDNA strands (from the original two). These are denatured again at approximately 94 degrees.

4. Again, the primers are annealed at a suitable temperature (somewhere between 50 and 60 degrees)

5. Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand. There are now eight cDNA strands

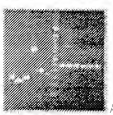
6. Again, the strands are denatured by raising the temperature to 94 degrees and then the primers are annealed at 60 degrees

7. The temperature is raised and the polymerase copies the eight strands to sixteen strands

8. The strands are denatured and primers are annealed

9. The fourth cycle results in 32 strands

10. Another round doubles the number of single stands to 64. Of the 32 double stranded cDNA molecules at this stage, 75% are the same size, that is the size of the distance between the two primers. The number of cDNA molecules of this size doubles at each round of synthesis (exponentially) while the strands of larger size only increase arithmetically and are soon a small proportion of the total number of molecules.



An agarose gel (1% Trevigel) stained with ethidium bromide and illuminated with UV light which causes the intercalated stain to fluoresce. The central lane shows markers that increase in size by 100bp. To the left are different PCR products using different primer pairs. To the right are control reactions using primers for actin

After 30 to 40 rounds of synthesis of cDNA, the reaction products are usually analyzed by agarose gel electrophoresis. The gel is stained with ethidium bromide

POWERPOINT

The above figures may be found in an animated PowerPoint presentation here.

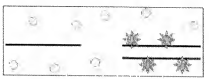
TUTORIAL

A Flash tutorial on PCR is here

This type of agarose gel-based analysis of cDNA products of reverse transcriptase-PCR does not allow accurate quantitation since ethidium bromide is rather insensitive and when a band is detectable, the exponential stage of amplification is over. This problem will be addressed in more detail below.

Ethidium bromide is a dye that binds to double stranded DNA by interpolation (intercalation) between the base pairs. Here it

fluoresces when irradiated in the UV part of the spectrum. However, the fluorescence is not very bright. Other dyes such as SYBR green, which are much more fluorescent than ethidium bromide, are used in real time PCR.



SYBR Green fluoresces brightly only when bound to double stranded DNA

In this presentation, we shall be using SYBR green to monitor DNA synthesis. SYBR green is a dye that binds to double stranded DNA but not to single-stranded DNA and is frequently used in real-time PCR reactions. When it is bound to double stranded DNA it fluoresces very brightly (much more brightly than ethidium bromide). We also use SYBR green because the ratio of fluorescence in the presence of double-stranded DNA to the fluorescence in the presence of single-stranded DNA is much higher that the ratio for ethidium bromide. Other methods can be used to detect the product during real-time PCR, but will not be discussed here. However, many of the principles discussed below apply to any real-time PCR reaction.

Now let us turn to real time PCR and, first, to why it was developed

REAL TIME PCR

As we noted above, normal reverse transcriptase PCR is only semi-quantitative at best because, in part, of the insensitivity of ethidium bromide. Thus real time PCR was developed because of:

- The need to quantitate differences in mRNA expression
- The availability of only small amounts of mRNA in some procedures such as in the use of:

    --- cells obtained by laser capture micro-dissection

    --- small amounts of tissue

    --- primary cells

    --- precious reagents

PROTOCOLS

There are a variety of methods for the quantitation of mRNA. These include:

Ribonuclease Protections Assay

Northern Blot

--- northern blotting

--- ribonuclease protection assays (RPA)

--- *in situ* hybridization

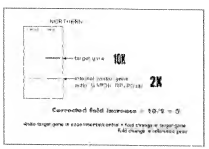
--- and PCR

PCR is the most sensitive method and can discriminate closely related mRNAs. It is technically simple but, as mentioned above, it is difficult to get truly quantitative results using conventional PCR.

Northern blotting and RPAs are the gold standards, since no amplification is involved, whereas *in situ* hybridization is qualitative rather than quantitative.

Techniques such as Northern blotting and RPAs work very well, but require more RNA than is sometimes available. PCR methods are therefore particularly valuable when amounts of RNA are low, since the fact that PCR involves an amplification step means that it is more sensitive.

In contrast to regular reverse transcriptase-PCR and analysis by agarose gels, real-time PCR gives quantitative results. An additional advantage of real-time PCR is the relative ease and convenience of use compared to some older methods (as long as one has access to a suitable real-time PCR machine).



So how do we use real-time PCR to quantitate the amount of DNA or cDNA?

The first two calculation methods for real-time that we are going to focus on are equivalent to the calculations that we usually perform when we do a northern blot. The image at the left shows a virtual northern blot with two lanes, one with RNA from control cells, the other with RNA from the experimental sample (e.g. drug treated cells). For the sake of argument, let's say that there is 10 times the amount of signal in the experimental sample compared to the control sample for the target gene. This could mean that expression of the gene has increased 10-fold in the experimental cells or it could mean that there is simply 10 times as much RNA in the experimental lane; in other words we have a loading artifact. To check for this we usually do a so-called 'Loading Control' in which the blot is probed for expression of a gene which does not change. In this case, let's say that the loading control shows that there is twice as much RNA in the experimental lane. This means that the real change in the target gene is 10/2 = 5 fold.

We can express this in a more general fashion:

ratio target gene expression (experimental/control) = $\frac{\text{fold change in target gene expression (expt/control)}}{\text{fold change in reference gene expression (expt/control)}}$

This brings us to the topic of standard or reference genes. A gene that is to be used as a loading control (or internal standard)

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,590,000,000
34	1,590,000,000

should have various features.

- The standard gene should have the same copy number in all cells
- It should be expressed in all cells
- A medium copy number is advantageous since the correction should be more accurate

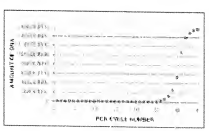
However, the perfect standard does not exist; therefore whatever you decide to use as a standard or standards should be validated for your tissue - If possible, you should be able to show that it does not change significantly in expression when your cells or tissues are subjected to the experimental variables you plan to use.

Commonly used standards are:

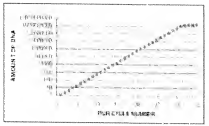
- Glyceraldehyde-3-phosphate dehydrogenase mRNA
- Beta actin mRNA
- MHC I (major histocompatibility complex I) mRNA
- Cyclophilin mRNA
- m RNAs for certain ribosomal proteins e.g. RPLP0 (ribosomal protein, large, P0). This is also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0.
- 28S or 18S rRNAs (ribosomal RNAs)

Now we need to think about the nature of the PCR reaction to understand real time QUANTITATION. The amount of DNA theoretically doubles with every cycle of PCR, as shown at the left. After each cycle , the amount of DNA is twice what it was before, so after two cycles we have 2 X 2 times as much, after 3 cycles - 2 X 2 X 2 times as much or 8 (2^3) times as much, after 4 cycles 2 X 2 X 2 X 2 times as much or 16 times (2^4) as much. Thus, after N cycles we shall have 2^N times as much.

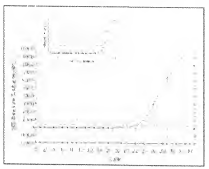
But, of course, the reaction cannot go on forever, and it eventually tails off and reaches a plateau phase, as shown by the figures in red.



If we plot these figures in the standard fashion (left). We cannot detect the amplification in the earlier cycles because the changes do not show up on this scale. Eventually you see the last few cycles of the linear phase (pink) as they rise above the baseline and then the non-linear or plateau phase (red) - Actually this starts somewhat earlier than is shown here.

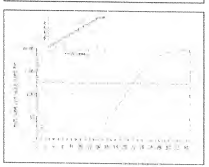


However, if we plot these values on a logarithmic scale, we can see the small differences at earlier cycles. In real time PCR we use both types of graph to examine the data. Note that there is a straight line relationship between the amount of DNA and cycle number when you look on a logarithmic scale. This is because PCR amplification is a exponential reaction.

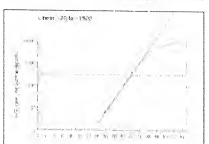


In the following discussion, the results shown will be those obtained using a Bio-Rad ICycler real-time PCR instrument; however, analysis with other machines is similar.

Here is a real time PCR trace for a single well on a 96-well plate; cycle number is shown along the X-axis and arbitrary fluorescence units (actually these are fold increase over background fluorescence) are shown on the Y-axis. You can see that this mimics our theoretical graph (inset) - except that the transition to the plateau phase is more gradual. This experiment - and everything we are going to discuss - was done with SYBR Green, which has very low fluorescence in the absence of double stranded DNA and very high fluorescence in the presence of double stranded DNA.

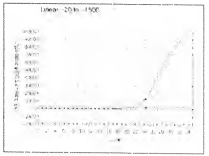


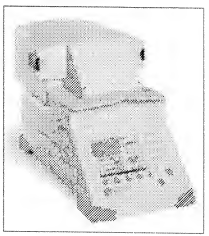
Here is the same real time PCR trace shown on a logarithmic scale - again it mimics our theoretical curve (inset).



As we saw with the theoretical curves, you should get a straight line relationship in the linear part of the PCR reaction. In this case the reaction is linear from ~20 to ~1500 arbitrary fluorescence units.

If we look at the same region on a regular scale, we see the linear part is, in fact, the very early part of the curve. Note that it is **NOT** the region which looks linear in this graphical view. This is a very important point in real-time PCR because we wish to examine the reaction while it is still in the linear phase.





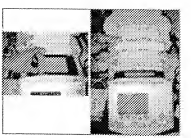
Thus Real Time PCR is a kinetic approach in which you look at the reaction in the early stages while it is still linear. There are many real time machines available and the one shown at the left is the ICycler® from BioRad. The lid slides back to accommodate samples in a 96-well plate format. This means that we can look at a lot of samples simultaneously. The machine contains a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. As DNA is synthesized, more SYBR Green will bind and the fluorescence will increase.

LINKS

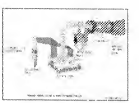
BioRad RT-PCR

Roche Light Cycler

The real-time machine is connected to a computer and software on the computer is needed to run the real time PCR machine in real-time mode

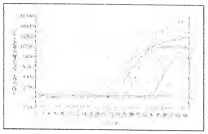


The plate is loaded and the lid is closed.



Optical detection system layout of BioRad iCycler® Image adapted from BioRad

So how do we measure differences in the concentration of DNA or cDNA? This graph shows a series of 10-Fold dilutions of a sample of DNA, and as we dilute the sample, it takes more cycles before the amplification is detectable. The dark blue line



here is the same sample that we have been following all along.

Note that although the reactions show a series of equally-spaced curves in order of dilution as they cross the orange line, they are rather variable when we look at the upper parts of the curve. Thus, if we stopped all these reactions at, for example, 33 cycles and analyzed the products on an agarose gel, it would indicate that the blue, red and purple reactions had the same amount of amplification, even though the reactions shown by the purple and red lines differ by a factor of 100 in the amount of DNA. This emphasizes why ethidium bromide-stained gels are **not** quantitative and, if used to measure cDNA in PCR reactions, can give erroneous results.

Thus, as we have emphasized, quantitation of the amount of cDNA in the original sample must be done where the amplification is exponential and, as we saw above, this is at the very beginning of the upturn of the curve and not in what **appears** to the linear region of the curve. In real time PCR, we measure the cycle number at which the increase in fluorescence (and therefore cDNA) is exponential. This is shown by the orange horizontal line in the figure (known as the threshold) and is set by the user. The point at which the fluorescence crosses the threshold is called the **Ct**.

It should also be noted that samples that differ by a factor of 2 in the original concentration of cDNA (derived from mRNA) would be expected to be 1 cycle apart. Thus samples that differ by a factor of 10 (as in our dilution series) would be ~3.3 cycles apart.

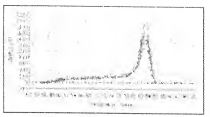
WHAT IS BEING AMPLIFIED IN OUR REACTION?

In real-time PCR using SYBR green binding to amplified cDNA, we are simply measuring the fluorescence increase as the dye binds to the increasing amount of DNA in the reaction tube. We hope that this increase in fluorescence is coming from the DNA that we wish to measure but some of the signal could come from DNA other than that which we are trying to amplify. Is there any way to check that the correct fragments were amplified? One way to do some checking of the products is to do a melting curve.

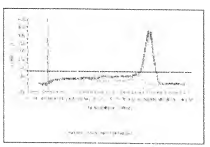
The real-time machine not only monitors DNA synthesis during the PCR, it also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short). All PCR products for a particular primer pair should have the same melting temperature - unless there is contamination, mispriming[1], primer-dimer[2] artifacts, or some other problem. Since SYBR green does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature. After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm). At the left are the melting curves for the samples on the previous picture; a primer-dimer artifact would give

a peak with a lower melting temperature (because it is such a short DNA).

If the peaks are not similar, this might suggest contamination, mispriming, primer-dimer artifact etc. You need to be sure that the only thing you detect with SYBR green is the thing you *want* to detect; that is a specific DNA fragment corresponding to the size predicted from the position of the primers on the cDNA (if you are looking at mRNA) or the genomic DNA, plasmid DNA, etc (according to what your target DNA is).



[1] *Mispriming: PCR products made due to annealing of the primers to complementary, or partially complementary sequences on non-target DNAs.*
[2] *Primer-dimer artifacts: the primers can sometimes anneal to themselves and create small templates for PCR amplification - these are the so-called primer-dimer artifacts.*



In this melting curve, all samples were run with the same primer pair, but the sample which contained no DNA (the red line) shows a melting curve with a lower Tm that the other samples; this is probably due to a primer-dimer artifact. With the SYBR green method, primer dimer artifacts are a problem since you are measuring total DNA synthesis and you need to be sure that you are measuring a Ct due to the real target for amplification. Fortunately, there are no signs of primer dimer artifacts in samples containing DNA in the graph at the left. However, this does stress the importance of primer design when using SYBR green.

So let's get back to the kinetics of SYBR green incorporation in our series of 10-fold dilutions. Here are the data on an arithmetic scale.

This shows the same data in the previous picture but on a logarithmic scale. The even spacing of the reactions is now much more obvious. What the software measures for each sample is the cycle number at which the fluorescence crosses the arbitrary line, the threshold, shown in orange. This crossing point is the Ct value. More dilute samples will cross at later Ct

values.

As we saw, it is important that the threshold should be in the linear part of the reaction - this is easier to see in the logarithmic view, where it should be no more than half way up the linear part; in the regular view, the threshold will be close to the bottom of the curve. However, the threshold should be high enough that you are sure that reactions cross the line due to amplification rather than noise. We find that if the plateau values are 4000 to to 15000, a threshold of 300 usually works well.

We use the same threshold for all the samples in the same experiment on the same plate.

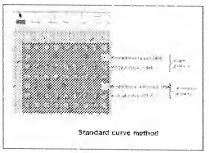
We can plot the Ct values for the dilutions against concentration - the result is a linear graph. It should have an excellent correlation coefficient (certainly more than 0.990).

QUANTITATION OF mRNA LEVELS USING REAL TIME PCR

STANDARD CURVE METHOD

There are several methods to quantitate alterations in mRNA levels using real time PCR. Let's look at the standard curve method first.

Here is an example of how we could set up a plate if we were using the standard curve method. By clicking on one of the symbols in the top line and then clicking on one of the wells in the plate diagram, we can define whether samples contain a



standard (circles) or an unknown (squares) or negative control samples containing water instead of DNA (-) and whether we have single wells containing the same sample or duplicate or triplicate wells (in which case the duplicate or triplicate well are assigned the same number). The software also allows you to define your dilution factors for the standard curves, give them names, etc. The negative controls are to check that your primers and Taq polymerase/SYBR green PCR mixes are not contaminated. They also allow you to determine if your primers can form primer-dimer artifacts which are most readily seen when there is no appropriate DNA for amplification (as shown above).

In this case we have extracted RNA from control cells (C) or cells treated in some special fashion (e.g. drug treated cells) - here designated (E) for experimental. These were then copied into cDNA using reverse transcriptase. We have set up the plate so that there is a standard curve for the loading control (or reference gene) and also one for the gene of interest whose expression we think may change under the experimental conditions (the so-called target gene). We normally just do a single point for each dilution of the standard curve since this gives a series of points which fit very well to a straight line. We usually do the cDNA samples in triplicate - so each sample will be done in triplicate for each gene.

You tell the software which dilution curve you want to use, and which unknowns you want it to quantitate using that curve. You do not actually give it a real copy number - just start at some arbitrary number.

The machine will report the copy number or amount of DNA in each of your unknown samples and even average this for you. If you take the average of the copy number in the experimental sample, divide it by the average copy number in the control sample, this will give the fold change in the target gene. You have now calculated the upper value in the "Northern" formula we derived earlier on.

Note the excellent fit of the standard curve data to a straight line - a perfect fit would have a correlation coefficient of 1.000 and here the correlation coefficient is 0.999.

Similarly, you can select the wells in which you amplified the reference gene and determine the relative amounts in the experimental sample compared to the control. This will give you the bottom value in the "Northern formula" derived earlier.

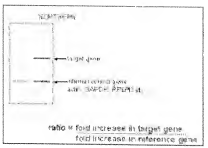
Now that you have both values, you can divide the target gene value (purple) by the reference gene value (blue) and obtain the ratio of the target gene in the experimental sample relative to the control sample, corrected for the reference gene (loading control).

This method will therefore give the fold changes in the target and reference genes - so we can calculate a fold change corrected for any variations in the reference gene just as one would do for a northern blot.

The disadvantage of this method is that you need a good dilution curve for both standard and reference genes on every plate - which would be at least 16 extra wells (including negative controls). If there is any problem with either dilution curve, the data cannot be analyzed, or a suboptimal curve has to be used. Thus, we prefer to determine efficiency accurately (on multiple

days) and then take an average of multiple results and use these separately - this makes experiments simpler but you need to think a bit more about the maths of calculating the results because this time the machine does not do it for you.

We find that the standard curves are highly reproducible if you use a supplier who provides a mix with stabilizer(s) for SYBR green.



PFAFFL METHOD

M.W. Pfaffl. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001, 29(9):e45.
M.W. Pfaffl, Nucleic Acids Research 2001 29:2002-2007

abstract
pdf file

LINKS
Gene Quantification
Dr Michael Pfaffl

So is there a way to do a similar calculation without doing an internal dilution curve each time? The answer is yes, we use the simple method developed by M.W. Pfaffl. However, to talk about this, we need to delve a bit more into the PCR reaction and examine the effects of the efficiency of amplification.

EFFECTS OF EFFICIENCY

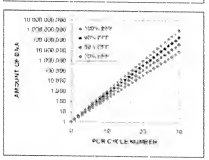
So first of all, let's discuss the significance of efficiency

Here is a series of calculations showing how much the DNA will be amplified if you have different efficiencies. For 100% efficiency, there will be a doubling of the amount of DNA at each cycle, for 90% the amount of DNA will increase from 1 to 1.9 at each cycle, so the factor is 1.9 for each cycle, and similarly for 80% and 70% it will be 1.8 and 1.7. Notice that a small difference in efficiency makes a lot of difference in the amount of final product. Each 10% lowering results in less than 25% of the previous column after 30 cycles.

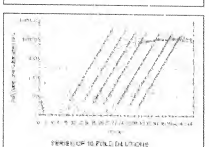
From this you can see that after 10 cycles the increase in DNA will be 2^{10}, if the efficiency is 100% (each cycle results in twice as much DNA), or 1.9^{10} if each cycle results in 1.9 times as much DNA - or to generalize, after n cycles, it will be [efficiency]^n.

There is some ambiguity in how people define efficiency. Some people say that if you copy 90% of your DNA in a cycle, so that you end up with 1.9 times as much, the efficiency is 1.9, and this is the definition that the Pfaffl equation uses. Other people say that the efficiency is actually 0.9 since one makes 0.9 times as much. If you use this definition the fold increase will be [1 + efficiency] ^{n}. We shall use the Pfaffl definition so we won't have to keep adding the '1'.

This shows the effect of changes in efficiency graphically. You can see that changes in efficiency have a major effect on the Ct value. Note also that changes in efficiency change the slope when you use the logarithmic scale.



Since the lines diverge at higher thresholds, lower thresholds will minimize the error due to small changes in efficiency. If a reaction has an inhibitor of PCR in it that reduces the efficiency, the slope will be different from unaffected reactions when you look at the results using the logarithmic scale. Thus, if you do triplicate reactions and one has a bad slope, you should drop that well from the analysis.



Here are the data from our dilution curve. If you are looking at efficiencies, you want to be sure that every time you do the PCR for the same gene you have the same slope since this is a measure of efficiency - in this case you can see that all the samples are reasonably close (the lines are all parallel). If there is a difference in slope of one of your samples, it implies a problem in that tube (PCR inhibitor, problems with the enzyme, etc).

SERIES OF 10-FOLD DILUTIONS

We can plot the Ct values for each of the dilutions against concentration - the result is a linear graph. It should have an excellent correlation coefficient (more than 0.990). The slope of this graph is a measure of efficiency, and can be readily used to calculate efficiency - but we shall not go into the math because the current version of the ICycler software does this for you.

QUALITY CONTROL - EFFICIENCY CURVES

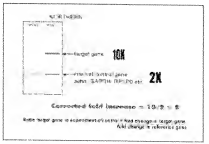
Here is a list of the criteria we apply in the lab before we accept the data for efficiency from a dilution curve

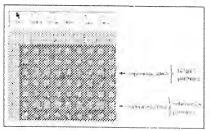
- use PCR baseline subtraction (not curve fitting default option)
- set the threshold manually to lab standard
- check all melting curves are OK
- check slopes are parallel in log view
- delete samples if multiple dilutions cross line together (usually at dilute end of curve)
- delete samples if can detect amplification at cycle 10 or earlier
- make sure there are 5 or more points
- check that correlation coefficient is more than 0.990

If you apply the top 7 criteria, the correlation coefficient is usually not a problem - we routinely get values of 0.998 and above.

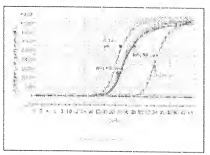
We find that the 'PCR base line subtracted curve fit' option (blue line in box at the top left), which is the default analysis mode in the current version of the ICycler program (3.0a) does not give such good results as the 'pcr base line subtracted' option. So we always use the latter.

So how does the Pfaffl method work? Again our approach is based on the "Northern equation".

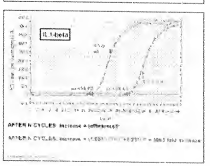




Here is the plate set up for this method. Note that this time we do not have to include a standard curve on every plate.



Here are the results from an experiment in which the target gene was IL1-beta and the reference gene was RPLPO. In this experiment, we investigated what happens when cells from the eye come in contact with vitreous humor. RNA was extracted from control (con) or vitreous-treated (vit) cells, and copied into cDNA. IL1-beta and RPLP0 assays were done in triplicate on cDNAs from both control and vitreous-treated cells.
We shall start by looking at the top part of the "Northern equation" - that is determining the fold change in IL1-beta in vitreous compared to control.



In the picture at the left, we are just looking at the results from the wells containing the IL1-beta primers. If we look at the difference in Ct values between the control and vitreous samples, we see there is an 11.60 cycle difference. Earlier on we derived the equation that the change in amount of DNA after n cycles is equal to the efficiency to the power of n. We independently do multiple serial dilution curves on multiple days to determine an average efficiency and we can then plug that value into the formula. Note that when determining the difference in the Ct values (sometimes known as the 'delta Ct'), we subtract the vitreous from the control value - this is so that increases will have a positive result and decreases a negative result for the delta Ct value.

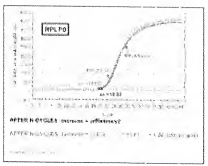
If the amount of RNA is less in the vitreous sample, the delta Ct would have a negative value and the change would have a value of less than 1.00. So, if there was half as much target mRNA the value for the change would be 0.5.
These calculations are easily done if you have an Excel spreadsheet set up (see later).

Thus from the data in this experiment we find.

Fold increase in target gene (IL-1 beta)

AFTER N CYCLES: increase = (efficiency)

$$\text{AFTER N CYCLES: increase} = (1.93)^{29.03-18.03} = 1.93^{11.00} = 2053 \text{ fold increase}$$



We had previously shown that the efficiency of the IL-1 beta primers was 93%

Here we are just looking at the RPLP0 data. Note, that, as expected for a good reference gene, there is not much difference between the two RNA samples with regard to their levels of RPLP0 mRNA. The same amount of total RNA was used for reverse transcription of both RNAs, and the same amount of each reverse transcription reaction was used for real-time PCR. Since the Ct values are so close and the ratio of the reference gene in the two samples is close to one, this suggests that RPLP0 is a good reference gene for these experiments.
Of course this method requires that you have determined the efficiency for your primers (87% in the case of the RPLP0 primers). We do this on multiple occasions, using the criteria for a good curve discussed above. We find that the values tend to be very reproducible if we use stabilized SYBR green mixes. If we change manufacturer or if the formulation is changed, the efficiency will need to be determined again.

Thus from the data in this experiment we find:

Fold increase in 'loading control' gene (RPLPO)

$$\text{AFTER N CYCLES: increase} = (\text{efficiency})^{\Omega}$$

$$\text{AFTER N CYCLES: increase} = (1.87)^{19.93-19.80} = (1.87)^{0.13} = 1.08 \text{ fold increase}$$

So we derived the change in IL1-beta mRNA (left panel) and in RPLP0 mRNA (right panel), and we now need to divide the change in target gene by the change in the reference gene as we would do in the 'Northern blot equation'.

As with the Northern Blot calculation, we need to correct for the loading control and so

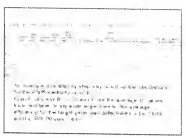
$$\text{ratio} = \frac{\text{change in IL1-B}}{\text{change in RPLP0}}$$

$$= \frac{2053}{1.08} = 1901$$

Spread sheet for Pfaffl equation calculations (Excel)

If we express this in a more general way as is shown below, we get a universal formula for doing these calculations - this is the formula in Pfaffl paper.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control-treated)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control-treated)}}}$$

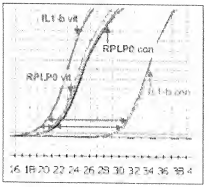


Here is a way to set up Excel spreadsheet to do the calculations for you. You do not actually need all the columns, you can just write the entire formula in one cell rather than subdividing it and spreading it across 5 cells as shown here (this was done so as to be clearer how the spreadsheet corresponds to the calculations we have discussed). However, this does allow you to scan readily for whether there is much change in the reference gene.

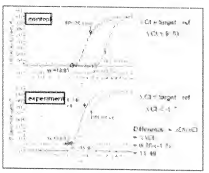
Delta-Delta CT METHOD (An approximation method)

This method was one of the first methods to be used to calculate real-time PCR results. However, as we shall see, it is an approximation method. It makes various assumptions, and to prove that they are valid is, in our opinion, more time consuming that doing a few extra efficiency runs for the Pfaffl method.

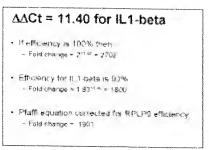
Let us look at the same data that we discussed before but, for the time being, we shall ignore the data from the standard 'loading control' gene. The difference between the control and treated samples for interleukin 1-beta is shown by the red line. If we know the efficiency for IL-1 beta and the cycle number, we could calculate the fold change in IL-1 beta - but there would be no loading control.



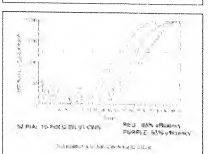
An approximate correction can be made for the loading control by calculating the difference between the IL1-beta Ct values and the RPLP0 values for the control samples, and then for the vitreous-treated samples (represented by the two green arrows in the picture). This makes an allowance for the fact that in the above case, there is slightly more mRNA in the vitreous-treated samples (since the RPLP0 comes up slightly earlier). This difference (or delta Ct value) is shown by the two green arrows. The difference between the two delta values represents the shift as will be seen in the next picture.



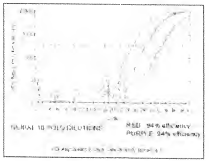
The difference between the two delta Ct values (delta delta Ct), represents the corrected shift of the IL1-beta. This is because, in this example, in the experimental sample the IL1-beta has moved to the left of the standard, it has a negative value, but in math subtraction of a negative value is equivalent to adding that value - which makes obvious sense if you look at the diagram. The total shift is equal to the two green arrows added together. If the experimental (vit) IL1-beta had shifted but remained to the right of the reference curve, the value would then be subtracted from the large green arrow to determine the shift.



Note how much different the results are when the correct efficiency is used rather than assuming 100%. The results are much closer to the ones calculated with the accurate method. However, note that the efficiency of the RPLP0 was never taken into account with this method. This method assumes that the efficiency of RPLP0 is so close to that of IL1-beta that it does not make much difference - or that the reference gene values are so similar for different RNA samples that the correction is so small that it would not be significant (it was only 1.08 fold when we calculated the change in RPLP0 cDNA for these same samples when discussing the Pfaffl method).



The problem with the delta delta ct is that it assumes that if you dilute the sample, the difference between the target gene and the reference gene will remain the same, so that the delta ct will be constant no matter how much you dilute the sample. However, as you can see in this figure, when we do a series of 10-fold dilutions the delta ct (difference between red and purple lines) does not remain constant - even with only 10% difference in efficiency. The purple is more efficient and gradually catches up.



However, if both primer sets have a similar efficiency, when we do a series of 10-fold dilutions the delta ct (difference between red and purple lines) remains constant (the slight variation for the last point here is probably because these samples were done as single wells per sample, if replicates had been included, the variation would be less).

SUMMARY OF EFFICIENCY($\Delta\Delta$Ct) METHOD

- This method assumes

  -- minimal correction for the standard gene, **or** that standard and target have similar efficiencies.

  * The $2^{\text{delta-delta Ct}}$ variant assumes efficiencies are both 100%

- This is an approximation method. But we need to validate that the assumptions are reasonably correct - For this we can do dilution curves to check that the DCts do not change
- The only extra information needed for the Pfaffl method is the reference gene efficiency. This involves only a little more work than validating the approximation method

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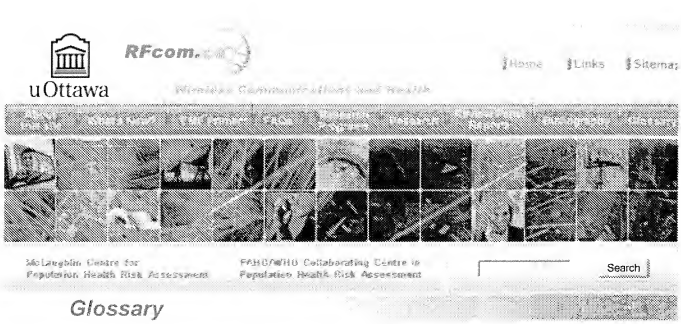

In the event that you encounter some unfamiliar terminology on our site or in any associated reports, we h this glossary of medical terms.

ACTH (adrenocorticotrophic hormone):A hormone secreted by the anterior pituitary gland that acts primarily on cortex, stimulating its growth and its secretion of corticosteroids. Its production is increased during times of stress.

Acoustic neuroma: A tumour of the auditoryc nerve.

Age-standardised: A rate which has been adjusted to minimise the effects of differences in age composition wher for different populations.

Alpha band: is the spectral component in the EEG signal, which falls between 8-13 Hz.

Aneuploidy: A genetically unbalanced condition in which a number of chromosomes for a organism that is not an exact multipl number. e trisomy 21 is a form of aneuploidy.

Apoptosis: Programmed cell death.

Association: Statistical dependence between two outcomes.

Atopic dermatitis (AD): A pruritic disease of unknown origin that usually starts in early infancy and is typified by pruritus, ecze xerosis (dry skin), and lichenification on the skin (thickening of the skin and increase in skin markings).

Autonomic nervous system: The portion of the nervous system concerned with regulation of the activity of cardia muscle, and glands. There are two main components, the sympathetic and the parasympathetic nervous systems.

Bcl-2 protein: Prototype for a family of mammalian genes and the proteins they produce. They govern mitochondrial outer mer permeabilization (MOMP) and can be either pro-apoptotic or anti-apoptotic.

Bias: Deviation of results or inferences from the truth, or processes leading to such deviation.

Binucleated: Having two nuclei.

Biological effects: A range of possible consequences, depending on the type and degree of cellular damage that may result fr external agent.

Blood-brain barrier: The barrier that exists between the blood and the cerebrospinal fluid, which prevents the pas substances from the blood stream to the brain. It is built up by endothelial cells lining the cerebral capillaries.

Caenorhabditis elegans: A microscopic (~1 mm) nematode (roundworm) that normally lives in soil.

Carcinogenesis: The production of a malignant new growth.

Case control study: A study that starts with the identification of persons with the disease (or other outcome) of int suitable control group of persons without the disease.

Causal relationship: The relating of causes to the effects they produce. Most of epidemiology concerns causality of causes can be distinguished. It should be clearly stated, however, that epidemiologic evidence by itself is insuffi causality.

Cell cycle: The cycle of cell growth, replication of the genetic material and nuclear and cytoplasmic division.

Chromatid: One of the usually paired and parallel strands of a duplicated chromosome, joined by a single centron

Chromosomes: The self-replicating genetic structures of cells containing the cellular dna that bears in its nucleoti linear array of genes.

Chromosome aberration: A deviation in the normal number of chromosomes or in their morphology.

Circadian: Pertaining to a period of about 24 hours; applied especially to the rhythmic repetition of certain phenom organisms at about the same time each day (circadian rhythm).

Clustering: An aggregation of relatively uncommon events or diseases with well-defined distribution patterns, in re place or both.

Cochlea: Snail-shapes structure in the inner ear that contains the organ of hearing.

Cognitive: Pertaining to cognition - that operation of the mind by which we become aware of objects of thought or includes all aspects of perceiving, thinking, and remembering.

Cohort study: A study in which a population (i.e., a cohort) is defined according to the presence or absence of a fa influence the probability of occurrence of a given disease or other outcome. The cohort is then followed to determi exposed to the factor are indeed at greater risk of the outcome.

Comet assay: An uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual cell.

Confidence interval: A range of values for a variable of interest e.g., a rate, constructed so that this range has a s probability of including the true value of the variable e.g. the reference to Dolk et al. mentions "an excess risk of 1.8 2.74)". This means that the estimated risk is 1.83, and there is a 95% probability that the "true" risk (if that could be within the range 1.22 2.74.

Confounding: The distortion of an apparent effect of an exposure on risk, brought about by the association with o can influence the outcome. For example, a study might suggest that alcohol intake is associated with a higher risk but this apparent relationship is seen because those who drink alcohol are also more inclined to smoke. When the taken into consideration, the relationship between alcohol intake and heart disease disappears.

Congenital: referring to conditions that are present at birth.

Control group: A sample in which a factor whose effect is being estimated is absent or is held constant, in order to provide a c

Cortisol: The major natural glucocorticoid hormone synthesised in the adrenal cortex. It affects the metabolism of protein, regulates the immune function, and has many other actvities.

Cross-sectional study: A study that examines the relationship between diseases and other factors, as they exist one particular time. The temporal sequence of cause and effect cannot necessarily be determined in this type of st

Cytochemistry: The study of the locations, structural relationships, and interactions of cellular constituents.

Cytogenetics: A branch of biology that deals with the study of heredity and variation by the methods of both cytology and gene approach to genetics, mainly involving microscopic studies of chomosomes.

DNA: Deoxyribonucleic acid; it constitutes the primary genetic material of all cellular material and the DNA viruses predominantly in the nucleus.

Dose-response relation: The relationship between the amount of exposure [Radiation) and the resulting changes in body func (response).

Double-blind trial: A procedure of blind assignment to study and control groups and blind assessment of outcome ensure that ascertainment of outcome is not biased by knowledge of the group to which an individual was assigne

Ecological study: A study in which the units of analysis are populations or groups of people, rather than individua

Electroencephalogram (EEG): a tracing of electrical activity arising from brain function

Electromagnetic sensibility: The ability to perceive the electromagnetic field (EMF) without necessarily developing health syn EMF exposure.

Endothelial: Pertaining to the layer of cells that lines the cavities of the heart and of the blood and lymph vessels.

Enzyme: A protein molecule that catalyzes chemical reactions of other substances without itself being destroyed c completing the reactions.

Epidemiology: The study of the distribution and determinants of health-related states or events in specified popul application of this study to control of health problems.

Ethyl methanesulfonate (EMS): A mutagenic, teratogenic, and possibly carcinogenic organic compound with formula $C_3H_8O_3$

Experimental study: A study in which conditions are under the direct control of the investigator.

Exposure: The amount of a factor to which a group or individual was exposed.

Exposure assessment: The process of measuring or estimating the intensity, frequency, and duration of human exposures to mobile phone radiofrequency field) currently present in the environment.

Fibroblast: Connective tissue cells which secrete an extracellular matrix rich in collagen and other macromolecules.

Fibrosis: The formation of fibrous tissue as a reparative or reactive process.

Free radical: A compound that carries an unpaired electron; such radicals are extremely reactive, with a very shor

FSH (follicle stimulating hormone): A hormone secreted by the anterior pituitary gland, which stimulates follicle ovary and estrogen production, and promotes the changes in the uterus characteristic of the first portion of the me the male, it stimulates spermatogenesis.

Gene Expression: The full use of the information in a gene via transcription and translation leading to production of a protein a appearance of the phenotype determined by that gene.

Genomics: A branch of biotechnology concerned with applying the techniques of genetics and molecular biology t mapping and DNA sequencing of sets of genes or the complete genomes of selected organisms using high-speed

Genotoxic: Damaging to DNA.

Glial fibrillary acidic protein (GFAP): The degenerative brain condition called Alexander disease is caused by mutation in GF acidic protein). The GFAP gene provides instructions for making GFAP protein, a member of the intermediate filament family th and strength to cells. Several molecules of GFAP protein bind together to form the main intermediate filament found in specializ called astrocytes.

GH (growth hormone): A hormone secreted by the anterior pituitary gland, which stimulates growth of the body. I effect on the metabolism of fat, carbohydrate, and protein.

Glial (or neuroglial): The supporting structure of nervous tissue.

Glioma: Usually used as a term to include all primary intrinsic neoplasms of the brain and spinal cord.

Gliosis: A process leading to scars in the central nervous system that involves the production of a dense fibrous network of ne cells) in areas of damage. Gliosis is a prominent feature of many diseases of the central nervous system, including multiple scl After a stroke, neurons die and disappear with replacement gliosis.

Healthy worker effect: Workers usually exhibit lower overall death rates than the general population, due to the fa severely ill and disabled are ordinarily excluded from employment. Death rates in the general population may be ir comparison if this effect is not taken into account.

Heat shock protein: Any of a group of proteins first identified as being synthesized in response to hyperthermia, h stresses, and believed to enable cells to recover from these stresses, perhaps by enabling recovery of gene expre a family of ubiquitously expressed proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are the cell's machinery for protein folding, and help to protect cells from stress.

Hematopoietic: Pertaining to or effecting the formation of blood cells.

Hormone: A chemical substance produced in the body by an organ, which has a specific regulatory effect on the a organs or cells.

Hippocampus: Area of gray matter extending the entire length of the floor of the temporal horn of the lateral ventr

Histology: That part of anatomy which deals with the minute structure, composition, and function of the tissues.

Hydrophilic: Readily absorbing moisture.

Immunocytochemistry: The application of immunochemical techniques (which use antibodies as chemical reage cytochemistry.

Incidence: The number of instances of illness commencing, or death occurring, during a given period in a specifie

In vitro: Observable in a test tube.

In vivo: Within the living body.

Ipsilateral: Located on or affecting the same side of the body. Opposite to contralateral.

Karyolysis: The destruction of a cells nucleus.

Karyorrhexis: Degeneration of the nucleus of a cell. There is contraction of the chromatin into small pieces, with obliteration of boundary.

Knockout: informal term for the generation of a mutant organism in which the function of a particular gene has be eliminated (a null allele).

Latency: The period of subclinical disease following exposure that ends with the onset of disease.

Leukemia: A progressive, malignant disease of the blood-forming organs, characterised by distorted proliferation a of leukocytes and their precursors in the blood and bone marrow.

- Lymphoblastic leukemia: Leukemia associated with overactivity of the lymphoid tissue. The acute type (ALL young children.
- Myeloid leukemia: Leukemia arising from myeloid tissue in which the granular, polymorphonuclear leukocyt precursors dominate.

Leukocyte: A white blood cell, specifically a colorless cell with a nucleus, found in blood and lymph. They can produce antibod through the walls of vessels to migrate to sites of injury, where they isolate and destroy dead tissue, foreign protein and bacteri

LH (luteinising hormone): A hormone secreted by the anterior pituitary gland, which with FSH promotes ovulatior androgen and progesterone secretion. In the male it stimulates the development and functional activity of testicula These cells produce male hormones, especially testosterone.

Lymphocyte: The white blood cell found in the blood, lymph, and lymphoid tissues, that are the body's immunolo cells and their precursors.

Lymphoma: Any neoplastic disorder of the lymphoid tissue.

- Hodgkin's disease or lymphoma: A form of lymphoma characterised by painless, progressive enlargement c nodes, spleen, and general lymphoid tissue. The characteristic histological feature is presence of Reed-Ste
- non-Hodgkin's lymphoma: A heterogeneous group of malignant lymphomas,the only common feature being the giant Reed-Sternberg cells.

Malignant: Malignant tumours have the ability to invade and destroy surrounding tissues and to spread to more di body (metastasis). Opposition to benign.

Melatonin: a hormone synthesised by the pineal gland. It is implicated in the regulation of sleep, mood, puberty, a

Meningioma: A benign, slow-growing tumour of the meninges, usually next to the dura mater.

Meta-analysis: A statistical technique for amalgamating, summarising, and reviewing previous quantitative research. By using wide variety of questions can be investigated, as long as a reasonable body of primary research studies exist. Selected parts c results of primary studies are entered into a database, and this "meta-data" is "meta-analyzed", in similar ways to working with descriptively and then inferentially to test certain hypotheses.

Monocytes: a relatively large mononuclear leukocyte that normally constitutes 3 – 7 % of the leukocytes in the cir and is normally found in lymph nodes, spleen. Bone marrow, and loose connective tissue.

Metabolism: The sum of the processes by which a particular substance is handled in the living body.

Micronucleus: The smaller of two types of nuclei when more than one is present in a cell. Micronuclei are thought chromosomal damage.

Misclassification: Inaccuracies in how subjects are categorized by exposure or disease status.

Mitotic: Pertaining to mitosis.

Microarray: Sets of miniaturized chemical reaction areas that may also be used to test DNA fragments, antibodies, or proteins having immobilized target and hybridising them with probed sample. the color we get from the chip after hybridisation is then sc is analysed by a soft ware to find the expression level.

Micronuclei: Chromosome fragments that are not incorporated into the nucleus at cell division.

Mortality ratio: Actual deaths in a specified time period divided by the expected number, usually multiplied by 100

Multivariate analysis: A set of techniques used when the variation in several variables has to be studied simultan

Mutagenic: Inducing genetic mutation.

Neoplasia: The formation of a new and abnormal growth.

Neuroglial (or glial): The supporting structure of nervous tissue.

Neuron(e): A nerve cell. The basic unit of the nervous system, specialized for the transmission of electrical impuls

Neurotransmitter: Any of a group of substances, released by a presynaptic cell that, upon excitation, crosses the stimulate or inhibit the postsynaptic cell.

Neutrophil: a granulocyte that is the chief phagocytic white blood cell.

Neurotrophic factors: These substances are responsible for the growth and survival of neurons during development, and for r neurons. Neurotrophic factors also are capable of making damaged neurons regrow their processes in a test tube and in anima

Observational study: An epidemiologic study in situations where nature is allowed to take its course; changes or characteristic are studied in relation to changes or differences in other(s), without the intervention of the investigato

Occipital lobe: The part of the brain near to the occipital bone, at the back of the head.

Odds ratio: The ratio of two odds. It is used frequently in case control studies where it is the ratio of the odds in fa disease, if exposed, to the odds in favour of getting disease if not exposed.

Oncogene: A gene capable under certain conditions of causing the initial and continuing conversion of normal cell cells.

Oncogenicity: The capacity to cause tumours.

Otoacoustic emissions: Low-intensity sounds produced by the inner ear that can be quickly measured with a ser placed in the ear canal.

Oxidation: The act of oxidizing or being oxidized. Chemically it consists of the increase of positive charges on an of negative charges.

Oxygen radicals: A substituent group of chemical elements rich in oxygen but incapable of prolonged existence ir

Parietal lobe: The part of brain near to the parietal bone.

Personal exposimeter: A dosimeter for assessing individual RF exposure in an urban environment in a free-living individual.

Physiological: Normal; not pathological; characteristic of the normal functioning or state of the body.

Pineal gland, or pineal body: A small, somewhat flattened, cone-shaped organ in the epithalamus of the brain. It synthesis of melatonin.

Pituitary gland: It is located at the base of the brain and is attached by a stalk to the hypothalamus, from which it important nerve and blood supply. There are two lobes - the anterior, which secretes most of the hormones, and th stores and releases neurohormones that it receives from the hypothalamus.

Placebo: An inert medication or procedure.

Precautionary principle: The principle is precautionary measures to anticipate, prevent or minimize the causes d radiofrequency fields and mitigate its adverse effects. Where there are threats of serious or irreversible damage, la certainty should not be used as a reason for postponing such measures.

Prevalence: The number of instances of a given disease or other condition in a given population at a designated t

Protein expression: A subcomponent of gene expression. It consists of the stages after DNA has been translated into amino a are ultimately folded into proteins.

Proteomics: A branch of biotechnology concerned with applying the techniques of molecular biology, biochemistr analyzing the structure, function, and interactions of the proteins produced by the genes of a particular cell, tissue, including the organization of the information in databases.

Psychomotor: Of or relating to movement or muscular activity associated with mental processes.

Psychophysiology: The physiology of psychology, i.e. the basic processes underlying the functioning of the mind processes.

Rate ratio: The ratio of two rates in epidemiology, the ratio of the rate in the exposed population to the rate in the population.

Random errors: vary in a nonreproducible way around a limiting mean. These errors can be treated statistically b of probability.

Real-time PCR: A method of simultaneous DNA quantification and amplification. DNA is specifically amplified by polymerase c (PCR). After each round of amplification, the DNA is quantified.

Relative risk: The ratio of the risk of disease or death among the exposed to the risk among the unexposed.

REM sleep (Rapid eye movement sleep): The period of sleep during which the brain waves are fast and of low v activities such as heart rate and respiration are irregular. This type of sleep is associated with dreaming, mild invol jerks, and rapid eye movements. It usually occurs three to four times each night at intervals of 80 to 120 minutes, e lasting from 5 minutes to more than an hour. In an adult, about 20 percent of sleep is REM sleep.

Risk: The probability that an event will occur.

Risk perception: The significance assigned to risks by stakeholders. This perception is derived form the stakeholders= expres and concerns.

Regression on Order Statistics (ROS): An implementation of a Regression on Order Statistics (ROS) designed for multiply c chemistry data. The method assumes data contains zero to many left censored (less-than) values.

Selection bias: Error due to systematic difference in characteristics between those who are selected for study anc not.

Sham exposure: A control group used to simulate the same environmental conditions of exposed samples, but in radiation.

Statistical significance: Statistical methods allow an estimate to be made of the probability of the observed or gre association between factors. From this estimate, in a sample of given size, the statistical "significance" of a result c number that expresses the probability that the result of a given experiment or study could not have occurred purely by chance.

Subjective outcomes: Outcomes or symptoms that are difficult to quantify objectively (e.g., pain, headaches, slee

Synapse: The functional membrane-to-membrane contact of the nerve cell with another nerve cell.

Synergize: two or more agents or forces interacting so that their combined effect is greater than the sum of their individual effects. **Antagonize:** two or more agents or forces interacting so that one agent counteracts the effect of another agent. **Potentiate:** one agent promotes or strengthens a biochemical or physiological action or effect of another agent.

Systematic errors: Are reproducible and tend to bias a result in one direction. Their causes can be assigned, at le and they can have constant and variable components.

Temporal lobe: The part of the brain near the temporal bone, in the lateral region of the head.

Temporal relationship: In epidemiology, the timing of the relationship between a factor and an outcome. It is one used to assign causality to a relationship.

Teratology: The division of embryology and pathology that deals with abnormal development and congenital anon

Trophoblasts: Cells forming the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large par They are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg.

Tinnitus: Ringing of the ears.

TSH (thyroid-stimulating hormone): A hormone secreted by the anterior pituitary gland that promotes the growth stimulates the hormonal secretion of the thyroid gland.

Tumourigenic: Capable of causing tumours. Can refer either to a carcinogenic substance or agent such as radiati cells or to transformed cells themselves.

Tumor laterality: The preference in location of tumor in one portion of the body over other locations in the body.

Uveal melanoma: Cancer of the eye.

Vestibule of the ear: The cavity of the inner ear.

Vestibular system (balance system): The sensory system that provides the dominant input about movement and equilibrioce

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